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TITLE: Identifying Breast Tumor Suppressors Using in Vitro and in Vivo RNAi Screens

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CONTRACTING ORGANIZATION: University of Miami
                                                    Miami, FL 33136

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Cancer is caused by mutations in oncogenes and tumor suppressor genes, resulting in the deregulation of processes fundamental to the normal behavior of cells. The identification and characterization of oncogenes and tumor suppressors has led to new treatment strategies that have significantly improved cancer outcome. The advent of next generation sequencing has allowed the elucidation of the fine structure of cancer genomes, however, the identification of pathogenic changes is complicated by the inherent genomic instability of cancer cells. Therefore, functional approaches for the identification of novel genes involved in the initiation and development of tumors are critical. During this research period we conducted the first whole human genome in vivo RNA interference screen to identify functionally important tumor suppressor genes. Using our novel approach, we identified previously validated tumor suppressor genes including TP53 and MNT, as well as several novel candidate tumor suppressor genes including leukemia inhibitory factor receptor (LIFR). These results demonstrate the power of genome wide in vivo RNAi screens as a method for identifying novel genes regulating tumorigenesis.

15. SUBJECT TERMS
In vivo RNA interference screen, breast cancer, tumor suppressor, leukemia inhibitory factor receptor (LIFR)

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INTRODUCTION

The purpose of this study is to functionally identify novel breast tumor suppressor genes. We have used an RNA interference (RNAi) based, forward genetic approach to identify genes that suppress oncogenic transformation of normal human breast epithelial cells in vivo. The identification of these genes will improve the understanding of the causes of breast cancer, which may lead to therapeutic advancements for breast cancer prevention and treatment.

BODY

Objective 1: Identification of breast tumor suppressors using *in vitro* and *in vivo* RNAi screens (Months 1- 14)

Original plan: Partially transformed human mammary epithelial cells (HMLEs) and primary breast epithelial cells (BPLEs) will be used for an RNAi screen to identify breast tumor suppressors. HMLEs and BPLEs will be infected with the Expression Arrest GIPZ lentiviral shRNAmir library consisting of 70,000 shRNAmirs targeting the whole human genome. shRNAmirs that silence tumor suppressors will fully transform HMLEs and BPLEs. Fully transformed cells will be identified by their ability to proliferate in the absence of extracellular matrix and form tumors in immunodeficient mice. Sequencing will identify the integrated shRNAmirs capable of full transformation. Target genes silenced by the identified shRNAmirs are candidate breast tumor suppressor genes capable of suppressing breast epithelial cell transformation.

Task 1A: Obtain regulatory approval (Months 1-3): COMPLETE
Regulatory approval from the Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC) has been obtained for the proposed experiments (Appendices A and B).

Task 1B: Prepare GIPZ shRNAmir library (Months 4-5): COMPLETE
Seven pools containing 10,000 GIPZ shRNAmir constructs were replicated in PrimePlus competent *E.coli*. Plasmid DNA was extracted using the Qiagen HiSpeed Maxi Kit. Plasmid DNA was digested with SacII restriction enzyme and visualized on an agarose gel with uncut DNA for diagnostic quality control (Supporting data, Figure 1). Plasmid pools showed no indication of recombination and passed quality control. Each pool of GIPZ shRNAmir plasmid DNA was packaged into lentiviral particles for transduction. Supernatants containing GIPZ shRNAmir lentivirus were harvested for transduction of partially transformed breast epithelial cells.

Task 1C: Complete in vitro shRNAmir screens (Months 6-8): ATTEMPTED, NOT COMPLETED DUE TO TECHNICAL ISSUES
BPLE cells proved very difficult to culture, resulting in an inability to generate enough BPLE cells for either the in vitro or in vivo RNAi screens. Therefore work was continued with only the HMLE cells.

The read-out for the in vitro RNAi screens is anchorage independent growth detected by the ability to form colonies in soft agar. Our screen depended on the HMLE cells gaining the ability to form colonies in soft agar by shRNA silencing of a tumor suppressor gene. Unfortunately our control experiments demonstrated that HMLE cells transduced with GIPZ non-silencing control already have some capability to form colonies in soft agar (Supporting data, Figure 2). Therefore they are not suitable for conducting an in vitro RNAi screen as the rate of false positives caused by spontaneous colony growth would be unacceptably high.

We are currently conducting experiments to determine whether primary mammary epithelial cells cultured from murine mammary glands are suitable for performing an in vitro RNAi screen. Primary murine mammary epithelial cells will be harvested and transduced with GIPZ non-silencing control or oncogenic Ras and their ability to form colonies in soft agar will be assessed. We expect that GIPZ non-silencing control cells will fail to form colonies and oncogenic Ras transduced cells will gain the ability to form colonies. If this is the case, these cells will be used for an in vitro RNAi screen to identify tumor suppressor genes.

Task 1D: Complete in vivo shRNAmir screens (Months 9-12): COMPLETE
BPLE cells proved very difficult to culture, resulting in an inability to generate enough BPLE cells for either the in vitro or in vivo RNAi screens. Therefore work was continued with only the HMLE cells.

Each pool of lentiviral packaged GIPZ shRNAmirs was used to infect six flasks of HMLE cells. In addition, per pool six flasks of HMLE cells were infected with lentiviral packaged GIPZ non-silencing control. Cells were transduced at a multiplicity of infection of 0.3 to ensure each cell integrated a single shRNAmir. 1000 cells per shRNAmir construct were transduced based on optimization experiments that demonstrated 1000 transformed cells could generate tumors in severely immunocompromised NSG mice. Nontransduced cells were removed by puromycin selection. Following puromycin selection, stably transduced cells were xenografted into the fourth mammary fat pads of NSG mice. To control for the rate of spontaneous transformation and viral insertional mutagenesis, mice were injected on the contralateral side with an equal number of HMLE cells infected with GIPZ non-silencing control. Six mice were injected per pool (7 pools) for a total of 42 mice to screen the complete genome-wide library. Mice were monitored for tumor formation for up to 250 days. In 19 of the 42 mice, tumors developed in the mammary fat pad injected with HMLE cells infected with the GIPZ library but not in the contralateral mammary fat pad injected with HMLE GIPZ NSC cells (Supporting data, Figures 3 and 4). Genomic DNA was extracted from all 19 tumors.

Task 1E: Identification of transformative shRNAmirs (Months: 13-14): COMPLETE
To identify the shRNAmirs capable of fully transforming breast epithelial cells, integrated shRNA sequences were identified by PCR amplification and next generation deep sequencing (NGS) of genomic DNA from all 19 tumors (Supporting data, Table 1). Specifically, virally integrated gene-specific shRNAmir sequences were PCR amplified from genomic DNA using primers that also contain the sequences required for cluster generation using the Illumina Genome Analyser II (GAII) platform. After cluster generation, clusters were sequenced on the GAII, using a primer that is complementary to a sequence flanking the gene-specific region of each shRNAmir. The output from the GAII comprises the identity and frequency of short DNA sequences representing the gene-specific target sequences of each shRNAmir. Identified shRNAs silence candidate breast tumor genes capable of suppressing breast epithelial cell transformation.

To support the results from NGS, we TOPO cloned and Sanger sequenced shRNA PCR products from all 19 tumors (Supporting data, Table 2). These approaches yielded a number of similar results. Importantly, previously characterized tumor suppressor genes including TP53 (1, 2) and MNT (3, 4) were identified, validating our in vivo RNAi screening strategy. In addition, many other candidate tumor suppressors were identified.

Our in vivo screen identified two individual shRNAs (V2LHS_7397 and V2LHS_133982) targeting the Leukemia Inhibitory Factor Receptor gene (LIFR) (Supporting data, Table 1) in tumors derived from multiple mice, reducing the likelihood that LIFR represented a false positive

hit (5). In addition, we confirmed that the presence of LIFR shRNA in the tumors was not due to unusually high representation in the pre-xenografted HMLE cells (Supporting data, Figure 5), indicating that the identification of LIFR was unlikely to be an artifact. Analysis of screen hit gene lists with the STRING protein interaction database (string-db.org) highlighted the interaction between LIFR and Ciliary Neurotrophic Factor Receptor (CNTFR) (6), an additional in vivo screen hit (Supporting data, Figure 6). Analysis of multiple genome-wide RNAi screens in Drosophila melanogaster has demonstrated that the identification of multiple components within protein-protein interaction networks is indicative of true positive hits (7). Therefore the identification of CNTFR further emphasizes the likelihood that LIFR is a positive hit from the in vivo RNAi screen.

LIFR is a subunit of the heterodimeric receptor for leukemia inhibitory factor (LIF). LIFR is expressed in normal breast epithelium, suggesting that LIFR function may be important in the normal breast. Although important in normal breast physiology, a potential role for LIFR as a breast tumor suppressor has not been previously described. Evidence that the LIF pathway may be mammary tumor suppressive is provided by mouse models of LIF deletion, which have defects in mammary epithelial apoptosis during post-lactational involution (8).

LIFR is one of the most promising candidates from our in vivo RNAi screen. Our future work will determine whether LIFR and other candidates are clinically significant novel breast tumor suppressor genes.

KEY RESEARCH ACCOMPLISHMENTS
- Obtained regulatory approval for study
- Prepared shRNAmir library for use in screens
- Determined BPLE cells not suitable for screens due to poor growth
- Determined HMLE cells not suitable for in vitro screen due to high background rate of colony formation in soft agar
- Conducted in vivo RNAi screen with HMLE cells
- Sequenced integrated shRNAs from genomic DNA extracted from HMLE tumors
- Identified potential breast tumor suppressors including promising candidate LIFR

REPORTABLE OUTCOMES
Presentations
2010 Invited Speaker, "Think Tank 20" Breast Cancer Symposium
2010 Invited Speaker, 14th Annual Laura Evans Memorial Breast Cancer Symposium
2010 Poster Presentation, AACR 33nd Annual San Antonio Breast Cancer Symposium
2011 Invited Speaker, "Think Tank 21" Breast Cancer Symposium
2011 Invited Speaker, 15th Annual Laura Evans Memorial Breast Cancer Symposium
2011 Poster Presentation, AACR 102nd Annual Meeting
2011 Invited Speaker, BCRP/CDMRP Department of Defense Era of Hope Conference

Manuscripts
Iorns, E., et. al. Whole genome in vivo RNAi screening identifies the Leukemia Inhibitory Factor Receptor as a novel breast tumor suppressor. *In preparation.*

Funding applied for
Cancer Institute NSW Early Career Fellowship Grant
National Health and Medical Research Council Project Grant
National Health and Medical Research Council Career Development Fellowship

Bankhead-Coley Cancer Research Program New Investigator Research Grant
Department of Defense Breast Cancer Research Program (BCRP) Idea Award
Stanley J. Glaser Foundation Research Awards

Employment received
The principle investigator, Dr Elizabeth Iorns received a promotion to a faculty position as Research Assistant Professor at the University of Miami Department of Medicine.

CONCLUSION
The goal of this study is to use RNAi technology to rapidly discover and validate novel breast tumor suppressor genes. By examining the role of every human gene in breast tumorigenesis we have identified new and unanticipated tumor suppressor genes, including LIFR. Further characterization of LIFR tumor suppressor function may allow the translation of this knowledge into useful therapeutic strategies to improve the survival of breast cancer patients.

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UNIVERSITY OF
Miami
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May 20th, 2009

Dear Lippman,

Thank you for your application.

A review of the referenced/attached submission entitled **RNAi screen for novel breast tumor suppressors** was completed. The IBC agrees that the application meets criteria for review under Section IIID and is in accordance with the *NIH Guidelines* - http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html - for work involving recombinant DNA.

You may initiate the study upon receipt of this notification.

Please reference IBC #09-035 with questions about this submission.

Do let me know if there are any questions.

Ellen Kapsalis, Ph.D.

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Protocol Approval Letter

06-May-2010

Dear Dr. LIPPMAN,

The following animal use application was reviewed and approved by the University of Miami Institutional Animal Care and Use Committee (IACUC). If conditional approval was issued, please address the additional approval requirements outlined below.

Protocol Number:	10-103 NEW
Protocol Title:	Identifying Breast Tumor Suppressors Using in Vitro and in Vivo RNAi Screens
Protocol Sponsor:	DEPT OF DEFENSE
Protocol PI:	LIPPMAN, MARC E
Institution:	University of Miami
Date of Approval:	21-May-2010
Duration of Approval:	21-May-2010 to 20-May-2011
Prior Protocol, if any:	
Additional Approval Requirements, if any:	

The University of Miami has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is #A-3224-01, effective July 11, 2007. Additionally, as of July 11, 2007, the Council on Accreditation of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) has continued the University of Miami's full accreditation.

Sincerely,

Paul Braunschweiger

Paul G. Braunschweiger, PhD
Chairman, **Institutional Animal Care and Use (IACUC)**
Professor, Department of Radiation Oncology

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Annual Renewal Approval

05-May-2011

Dear Dr. LIPPMAN,

The following annual renewal was reviewed and approved by the University of Miami Institutional Animal Care and Use Committee (IACUC) as of the date below:

Protocol Number:	10-103 Renewal 02
Protocol Title:	Identifying Breast Tumor Suppressors Using in Vitro and in Vivo RNAi Screens
Protocol Sponsor:	DEPT OF DEFENSE
Protocol PI:	LIPPMAN, MARC E
Institution:	University of Miami
Date of Approval:	05-May-2011
Approval Period:	21-May-2011 to 20-May-2012

The University of Miami has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is #A-3224-01, effective July 11, 2007. Additionally, as of July 20, 2010, the Council on Accreditation of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) has continued the University of Miami's full accreditation.

Sincerely,

Sari Izenwasser, PhD

Chair, **Institutional Animal Care and Use (IACUC)**
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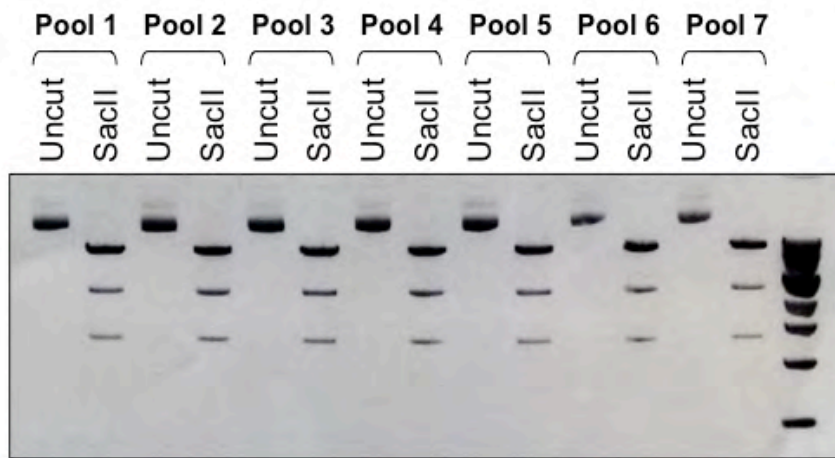
FIGURE 1

Figure 1. Diagnostic digest of GIPZ library pool DNA. Constructs are stable, no recombination products are visible. Expected bands: 8kb, 2.5kb, 1.25kb.

FIGURE 2
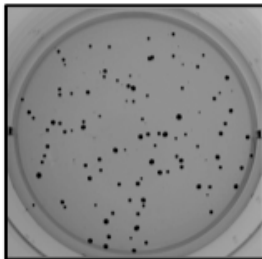
Figure 2. HMLE cells transduced with GIPZ non-silencing control (NSC) form colonies in soft agar. Representative photograph of crystal violet stained soft agar colony formation 21 days after culture of HMLE NSC cells.

FIGURE 3
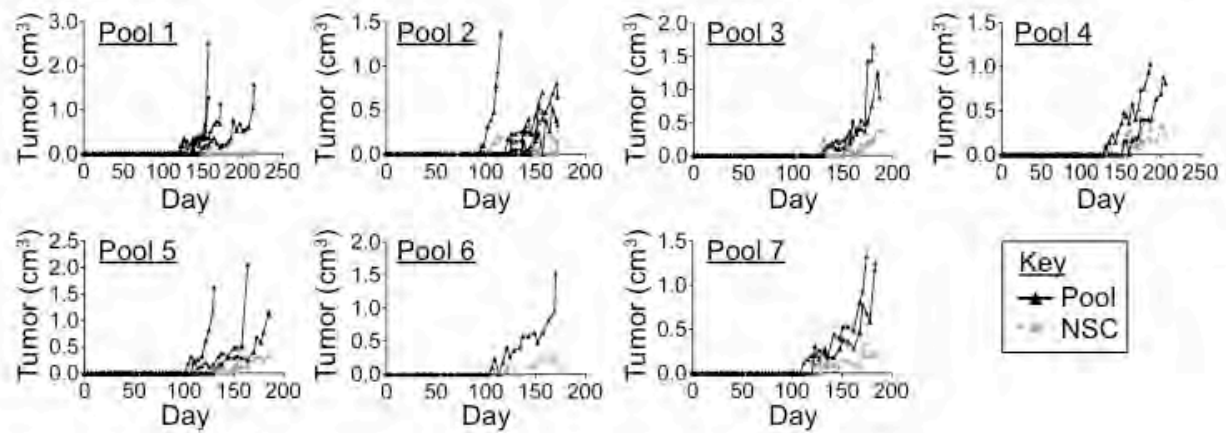
Figure 3. Volumes of tumors derived from HMLE cells infected with pools 1-7 of the Expression Arrest™ GIPZ shRNA library containing 10,000 GIPZ shRNA constructs (black) or GIPZ non silencing control (NSC) shRNA (grey). Tumors developed from HMLE cells infected with pool 1 but not GIPZ NSC in 4 of 6 mice, pool 2 but not GIPZ NSC in 5 of 6 mice, pool 3 but not GIPZ NSC in 2 of 6 mice, pool 4 but not GIPZ NSC in 2 of 6 mice, pool 5 but not GIPZ NSC in 3 of 6 mice, pool 6 but not GIPZ NSC in 1 of 6 mice, and pool 7 but not GIPZ NSC in 2 of 6 mice (total 19 of 42 mice).

FIGURE 4

Figure 4. Photographs of a representative mouse (left flank GIPZ NSC, right flank GIPZ pool 1) and a tumor derived from GIPZ pool 1 infected cells are shown, along with haematoxylin and eosin staining of tumor tissue derived from GIPZ pool 1 infected cells.

FIGURE 5
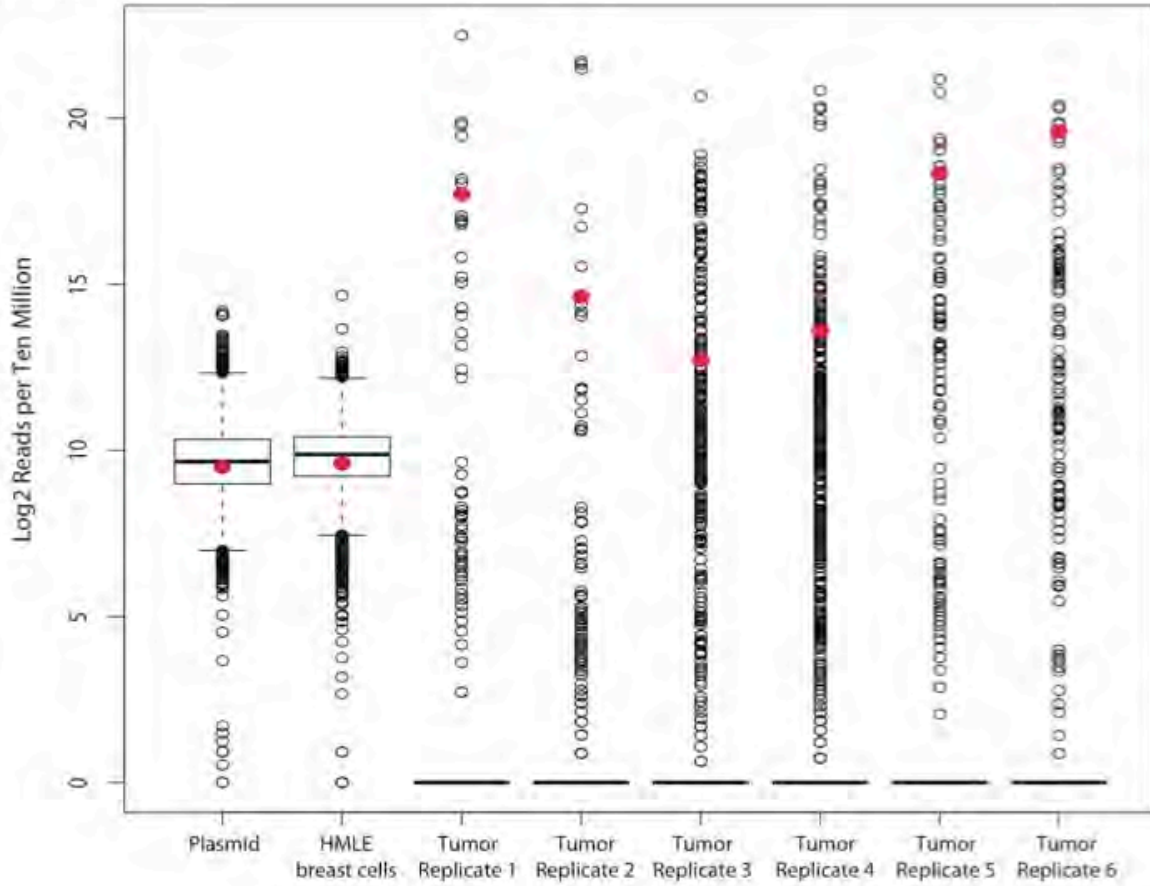
Figure 5. Boxplot depicting the distribution of next generation sequencing read counts (log2 transformed) for each shRNA in pool 1 of the genome-wide library. The frequency of each shRNA construct in pool 1 (containing LIFR shRNA) was estimated by next generation sequencing using either: (i) the original plasmid DNA pool used to generate pool 1 virus, (ii) genomic DNA from pre-xenografted HMLE cells transduced with pool 1 virus and, (iii) genomic DNA from tumors derived from HMLE cells transduced with pool 1 virus. Boxplots demonstrate that the representation of each shRNA construct (the relative frequency of each shRNA in the pool) was similar between the original plasmid and pre-xenografted HMLE cells. In both samples, the relative frequency of shRNA targeting LIFR (labeled in red) was close to the median representation for pool 1. In tumors derived from the transduced HMLE cells, only 5% of the original shRNAs were present (hence the first, second and third quartiles of the shRNA distribution being equal to zero) and this 5% included shRNA targeting LIFR. The original representation of LIFR shRNA in the plasmid and pre-xenografted HMLE cells (close to the median) suggests that the presence of this shRNA in the subsequent tumors is unlikely to be an artifact of an unusually high representation in the starting material.

FIGURE 6

Figure 6. Protein association network of candidate tumor suppressor genes identified from RNAi screen.

Table 1. List of candidate tumor suppressor genes identified by NGS of tumor integrated shRNAs.

| shRNA ID	Pool	Target 1	Target 2	Targets	Median sequence reads	% tumors present	shRNAs/ gene
V2LHS_133982	**3**	**LIFR**		**1**	**164141**	**100**	**2**
V2LHS_7397	**1**	**LIFR**		**1**	**120077**	**100**	**2**
V2LHS_132866	5	ATP6V1C1		1	57265	33	2
V2LHS_132867	4	ATP6V1C1		1	31071	100	2
V2LHS_93615	5	TP53	FXR2	2	499650	33	2
V2LHS_217	1	TP53	FXR2	2	3375	50	2
V2LHS_204961	4	CECR1		1	9421862	100	1
V2LHS_150832	4	CPA2		1	7377893	50	1
V2LHS_194392	2	TAS2R14		1	3654761	40	1
V2LHS_182911	1	PRODH2		1	2967566	33	1
V2LHS_285606	2	HDDC3	UNC45A	2	2501888	20	1
V2LHS_276657	2	PPAPR3		1	2009357	30	1
V2LHS_72117	5	TUBA3C		1	1967019	33	1
V2LHS_37616	5	UBE2K		1	1862995	33	1
V2LHS_181721	2	EFCAB5		1	1573073	30	1
V2LHS_202269	7	AC107883.1	COX5A	2	1348435	50	1
V2LHS_191885	5	FKBP1A	SDCBP2	2	1137629	33	1
V2LHS_118826	7	CMTM8		1	937313	50	1
V2LHS_156567	6	EXOC1		1	900149	100	1
V2LHS_175007	2	RNF121		1	883033	40	1
V2LHS_51176	5	FBXL7		1	841655	100	1
V2LHS_117561	2	L3MBTL2		1	827013	40	1
V2LHS_173142	1	GUCY1B2		1	688827	33	1
V2LHS_218555	1	ZBTB17		1	638940	33	1
V2LHS_46777	3	TAAR5		1	634677	100	1
V2LHS_47611	5	MAP4K2		1	623917	83	1
V2LHS_112635	7	BUB1B		1	618581	50	1
V2LHS_266098	1	AC010634.1		1	618470	33	1
V2LHS_152280	1	GPNMB		1	489561	33	1
V2LHS_213752	1	ASCC3		1	488422	33	1
V2LHS_131392	1	POLR2B		1	467182	33	1
V2LHS_257730	1	MCOLN2	SYDE2	2	464493	33	1
V2LHS_98652	1	MRP63	ZMYM2	2	459575	33	1
V2LHS_68041	3	DIS3L2	EIF4E2	2	452545	100	1
V2LHS_174587	2	PID1		1	411779	60	1
V2LHS_150703	1	CNTFR		1	387166	33	1
V2LHS_171793	1	UBE2N		1	353683	33	1
V2LHS_169903	5	PFKP		1	347281	33	1
V2LHS_58990	1	B3GALTL	N4BP2L1	2	343719	33	1
V2LHS_8705	1	WHSC1		1	317411	33	1
V2LHS_66509	3	DMTF1		1	316145	75	1
V2LHS_155301	5	FRMD4A		1	307011	33	1
V2LHS_155295	5	PACS1		1	292135	33	1
V2LHS_60727	5	USP22		1	269208	33	1
V2LHS_131441	1	PRIM2		1	263467	50	1
V2LHS_168960	1	CDA		1	230849	33	1
V2LHS_96376	6	WDFY3		1	217892	100	1
V2LHS_117997	1	PRAM1		1	208361	33	1
V2LHS_182675	5	KIAA1602	SENP1	2	204929	33	1
V2LHS_82602	6	HADHB	RAB10	2	192323	100	1
V2LHS_87361	4	MLST8		1	186159	100	1
V2LHS_139564	3	VPS13C		1	182346	75	1
V2LHS_99134	2	AC007639.1	C17orf28	2	169421	60	1
V2LHS_71132	5	ABI3BP	AC107297.1	2	166017	33	1

V2LHS_162802	6	OR51E2		1	165424	100	1
V2LHS_15884	1	SFT2D1		1	136056	33	1
V2LHS_263191	1	AC006222.1	SUFU	2	133628	33	1
V2LHS_112107	6	ATP1A1		1	129034	100	1
V2LHS_68676	1	AL353616.3	C9orf61	2	128434	33	1
V2LHS_73744	6	C7orf33		1	123787	100	1
V2LHS_234326	3	TMEM45A		1	121674	50	1
V2LHS_101877	6	AC114947.2		1	120067	100	1
V2LHS_110972	7	HUWE1		1	116783	50	1
V2LHS_246594	2	C22orf13		1	111406	60	1
V2LHS_259788	6	SFMBT2		1	104071	100	1
V2LHS_39993	2	MNT	TSR1	2	97502	20	1
V2LHS_69631	3	ANO4		1	94057	50	1
V2LHS_1160	1	PCTK3		1	93360	33	1
V2LHS_38291	6	NXPH1		1	92513	100	1
V2LHS_7031	1	CCR3		1	91259	33	1
V2LHS_259294	6	FAM177B		1	88434	100	1
V2LHS_48160	1	NDUFB10		1	80360	33	1
V2LHS_213497	3	TLE4		1	77232	75	1
V2LHS_48808	1	AL135798.2	IGSF3	2	76436	100	1
V2LHS_75122	1	TAAR8		1	70476	100	1
V2LHS_100143	3	KIF5C	LYPD6B	2	69814	50	1
V2LHS_94543	5	APAF1		1	62633	33	1
V2LHS_266450	4	OR5W2		1	59496	50	1
V2LHS_211996	5	TIAM2		1	57567	33	1
V2LHS_268505	3	AC007952.1	SLC5A10	2	57291	50	1
V2LHS_188289	2	CSH2		1	56757	80	1
V2LHS_17003	2	GCSH	PCDH1	2	53500	20	1
V2LHS_174170	5	TIPIN	Z97054.3	2	47393	33	1
V2LHS_102439	3	ADCY1		1	46096	50	1
V2LHS_28335	4	CD83		1	45878	50	1
V2LHS_43414	5	RALGPS2		1	45257	33	1
V2LHS_183847	5	Z98036.1		1	45117	50	1
V2LHS_262163	5	NEK1		1	36237	33	1
V2LHS_49523	2	GIF	OSBP	2	36223	20	1
V2LHS_23977	3	NUMB		1	35870	50	1
V2LHS_23264	3	CTSF		1	35419	50	1
V2LHS_28429	5	TNKS1BP1	UBE2L6	2	33927	33	1
V2LHS_121978	1	H2BFM	TMSB15B	2	33848	83	1
V2LHS_100226	1	C22orf37	LA16c13E4.2	2	32841	33	1
V2LHS_23622	5	AC061997.1		1	32002	33	1
V2LHS_19462	3	PRF1		1	30257	50	1
V2LHS_58054	1	POU4F1		1	29241	33	1
V2LHS_35209	3	MVP		1	24109	50	1
V2LHS_93049	1	PDE6A		1	23249	33	1
V2LHS_137892	5	ZNF611		1	22064	33	1
V2LHS_156659	2	KIF24		1	21963	70	1
V2LHS_131694	5	FAAH	TSPAN1	2	21601	50	1
V2LHS_17391	3	MYH13		1	21592	50	1
V2LHS_206583	2	ZNF330		1	21326	20	1
V2LHS_112759	1	AC034187.2	CAV3	2	20983	33	1
V2LHS_178044	2	C1orf94		1	20926	20	1
V2LHS_215569	5	LRRC16A		1	20767	33	1
V2LHS_239676	2	CYB5D1	DNAH2	2	20249	60	1
V2LHS_144730	5	SIDT1	TMPRSS7	2	20129	33	1
V2LHS_198745	4	ABL1		1	19901	50	1
V2LHS_172019	5	WNT5A		1	19276	33	1
V2LHS_137570	5	PDCD1LG2		1	17852	33	1
V2LHS_185093	5	AL353573.1		1	17665	33	1

V2LHS_113021	5	CHUK		1	17578	50	1
V2LHS_177716	1	C9orf3		1	16438	33	1
V2LHS_91177	5	RNF13		1	14746	33	1
V2LHS_276962	5	MGAT5		1	13816	50	1
V2LHS_178409	2	SLAMF9		1	13152	20	1
V2LHS_208669	5	WWTR1		1	12979	33	1
V2LHS_52284	4	AL603926.6	ZNF26	2	12438	50	1
V2LHS_246460	5	ZDHHC15		1	10654	50	1
V2LHS_44075	5	PITPNC1		1	10433	33	1
V2LHS_177944	5	CGNL1		1	9858	33	1
V2LHS_64338	5	NEUROG1		1	9706	33	1
V2LHS_231899	2	TMC3		1	9507	40	1
V2LHS_168877	5	CLASP2		1	9387	33	1
V2LHS_115250	1	AK3		1	7984	33	1
V2LHS_208512	5	NAB1		1	7855	33	1
V2LHS_261875	1	SPAG4		1	7339	83	1
V2LHS_201791	4	AARS	AP1G1	2	7097	50	1
V2LHS_285409	5	PPAPDC2		1	6814	33	1
V2LHS_135271	5	SLC6A13		1	6766	33	1
V2LHS_205126	1	C8orf80		1	5814	33	1
V2LHS_156003	5	ASF1B	RFX1	2	5366	33	1
V2LHS_200379	3	ASB15		1	5243	50	1
V2LHS_19602	7	TMEM170A		1	5016	50	1
V2LHS_60377	1	XIRP2		1	4505	50	1
V2LHS_236770	5	EFHD2	FHAD1	2	3811	33	1
V2LHS_180163	3	AC004797.1	NSF	2	3604	50	1
V2LHS_17187	4	ADAM20		1	3060	50	1
V2LHS_46512	4	CPSF3	DSC2	2	2985	50	1
V2LHS_172979	1	STOM		1	2930	33	1
V2LHS_93704	4	FCGR1A		1	2652	50	1
V2LHS_34729	6	AC048351.3	FGF12	2	1770	100	1
V2LHS_18791	5	KCND2		1	1494	33	1

Table 2. List of candidate tumor suppressor genes identified by TOPO cloning and Sanger sequencing of tumor integrated shRNAs.

shRNA ID	Pool	Tumor	Target	No. of clones	% of clones
V2LHS_75122	1	1	TAAR8	5	71
V2LHS_8292	1	1	CMKLR1	2	29
V2LHS_111807	1	2	BCL2	3	20
V2LHS_173142	1	2	GUCY1B2	2	13
V2LHS_33394	1	2	KRTCAP3	1	7
V2LHS_118666	1	2	IGSF10	1	7
V2LHS_121978	1	2	H2BFM	1	7
V2LHS_140316	1	2	RBM33	1	7
V2LHS_98652	1	2	MRP63	1	7
V2LHS_129460	1	2	AC091996	3	20
V2LHS_266098	1	2	AC034218	1	7
V2LHS_48808	1	2	AL135798	1	7
V2LHS_171793	1	3	UBE2N	4	40
V2LHS_261875	1	3	SPAG4	1	10
V2LHS_7397	1	3	LIFR	1	10
V2LHS_131441	1	3	PRIM2	1	10
V2LHS_151585	1	3	MATN1	1	10
V2LHS_250176	1	3	AC087897	1	10
V2LHS_58990	1	3	AL137247	1	10
V2LHS_20834	1	4	ITGA11	6	30
V2LHS_71628	1	4	TSC22D1	5	25
V2LHS_58310	1	4	PNOC	6	30
V2LHS_20955	1	4	NR_028509	1	5
V2LHS_117602	1	4	C1orf124	2	10
V2LHS_181721	2	1	EFCAB5	5	36
V2LHS_276657	2	1	LPPR1	3	21
V2LHS_188289	2	1	CSH2	2	14
V2LHS_175007	2	1	RNF121	2	14
V2LHS_26757	2	1	ADARB2	1	7
V2LHS_117561	2	1	L3MBTL2	1	7
V2LHS_39993	2	2	MNT	3	23
V2LHS_277663	2	2	RANBP1	2	15
V2LHS_193744	2	2	XR_016525	8	62
V2LHS_202124	2	3	XRCC4	5	36
V2LHS_132865	2	3	ATP6v1c1	3	21
V2LHS_156659	2	3	KIF24	2	14
V2LHS_12782	2	3	PDCD4	1	7
V2LHS_219636	2	3	MAPK10	1	7
V2LHS_215353	2	3	AL390715	1	7
V2LHS_238071	2	3	Unknown	1	7
V2LHS_262685	2	4	CLK2	12	57
V2LHS_284921	2	4	VPREB1	1	5
V2LHS_253420	2	4	AL359971	4	19
V2LHS_147291	2	4	Unknown	2	10
V2LHS_213735	2	4	LOC643563	1	5
V2LHS_225401	2	4	CG030	1	5
V2LHS_235027	2	5	OSBPL9	3	27
V2LHS_132865	2	5	ATP6v1c1	1	9
V2LHS_238638	2	5	C10orf76	2	18
V2LHS_162907	2	5	OR5M14P	1	9
V2LHS_28713	2	5	PGLS	1	9
V2LHS_177129	2	5	AC009137	1	9
V2LHS_167482	2	5	AC018608	2	18
V2LHS_132867	3	1	ATP6v1c1	3	100
V2LHS_39993	3	2	MNT	5	31

V2LHS_46777	3	2	TAAR5	3	19
V2LHS_68041	3	2	EIF4E2	4	25
V2LHS_66509	3	2	DMTF1	4	25
V2LHS_150832	4	1	CPA2	3	30
V2LHS_216696	4	1	CACNB4	1	10
V2LHS_245372	4	1	SMS	1	10
V2LHS_244347	4	1	AC068292	1	10
V2LHS_198745	4	1	ABL1	1	10
V2LHS_47040	4	1	ATP6v0e1	1	10
V2LHS_222702	4	1	AK098479	1	10
V2LHS_201113	4	1	JHDM1D	1	10
V2LHS_204961	4	2	HIRA	10	59
V2LHS_110904	4	2	AC010100	7	41
V2LHS_156659	5	1	KIF24	1	10
V2LHS_152028	5	1	MX1	1	10
V2LHS_207798	5	1	LOC646324	1	10
V2LHS_238142	5	1	AL359392	1	10
V2LHS_250919	5	1	USP35	1	10
V2LHS_210938	5	1	SULT1B1	1	10
V2LHS_54636	5	1	SR140	1	10
V2LHS_19384	5	1	RELN	1	10
V2LHS_50526	5	1	RPLP0P4	1	10
V2LHS_211667	5	1	POLR3G	1	10
V2LHS_275779	5	2	HIST4H4	1	14
V2LHS_24996	5	2	CA14	1	14
V2LHS_50258	5	2	XR_016873	4	57
V2LHS_142260	5	2	XR_017265	1	14
V2LHS_51176	5	3	FBXL7	8	44
V2LHS_47611	5	3	MAP4K2	2	11
V2LHS_246460	5	3	ZDHHC15	2	11
V2LHS_256533	5	3	PEX12	1	6
V2LHS_191885	5	3	FKBP1A	1	6
V2LHS_179595	5	3	ITGA1	1	6
V2LHS_183847	5	3	Z98036	1	6
V2LHS_182675	5	3	Unknown	1	6
V2LHS_34838	5	3	Unknown	1	6
V2LHS_73744	6	1	RNY4	1	10
V2LHS_30715	6	1	AC025035	1	10
V2LHS_120707	6	1	OR5J7P	1	10
V2LHS_114931	6	1	PRLH	1	10
V2LHS_259788	6	1	AL590095	1	10
V2LHS_205654	6	1	AC021150	4	40
V2LHS_131528	6	1	PTGS1	1	10
V2LHS_219840	7	1	CBR4	3	27
V2LHS_123243	7	1	KRT27	3	27
V2LHS_96369	7	1	RIMS1	1	9
V2LHS_90902	7	1	PIF1	1	9
V2LHS_149971	7	1	CRPP1	1	9
V2LHS_23683	7	1	AC126469	1	9
V2LHS_78374	7	1	AC083949	1	9
V2LHS_118826	7	2	CMTM8	1	10
V2LHS_265739	7	2	FGFR1OP2	1	10
V2LHS_165312	7	2	AMTN	1	10
V2LHS_219613	7	2	AL358937	1	10
V2LHS_146428	7	2	AC120305	1	10
V2LHS_285756	7	2	BTN2A1	1	10
V2LHS_36022	7	2	STK17A	2	20
V2LHS_20834	7	2	ITGA11	1	10
V2LHS_135243	7	2	TLR8	1	10